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TITLE OF THE INVENTION (280 characters max)

METHOD OF ENTRAPPING LOW MOLECULAR WEIGHT PROTEINS USING CHAPERONINS

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METHOD OF PAYMENT (check one)

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| ☒ A check or money order is enclosed to cover the Provisional filing fees | Provisional filing fee amount(s) | $ 160.00 |
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# METHOD OF ENTRAPPING LOW MOLECULAR WEIGHT PROTEINS USING CHAPERONINS.

## FIELD OF INVENTION

This field of invention relates to a method of entrapping low molecular weight proteins from fluid sample such as human serum using bacterial chaperonins immobilized on a solid surface.

## BACKGROUND OF THE INVENTION

It has been known that human serum proteins exist in nine order of magnitude difference in concentration. The twelve most abundant proteins contribute to more than 95 % of total protein constituents. Potentially interesting biomarkers comprise the remaining 5% of protein content. Attempts have been made to prefractionate serum proteins. Some of the current technologies for fractionation and purification of serum proteins are liquid chromatography MS-MS or SELDI (Surface Enhanced Laser Desorption Ionization). However, they either suffer from non-specific removal of associating proteins or their efficiencies are relatively low. The object of this invention is to entrap low molecular weight proteins.

## SUMMARY

According to the first aspect of this invention, we provide a method of entrapping low molecular weight proteins comprising: immobilizing a chaperonin on a surface; and capturing low molecular weight proteins of a denatured fluid sample with the chaperonin.

Preferably, the invention comprises of modifying the chaperonin by site-directed mutagenesis. In particular, the invention further provides a method of modifying apical domain of the chaperonin and thereby control spectrum of captured low molecular weight proteins.

In various embodiments, the fluid sample comprises of a serum, a cerebral spinal fluid, urine or nipple aspirant.

In one embodiment, the chaperonin is *E. Coli* GroEL. In another embodiment, the chaperonin is *T.th* GroEL.

In various embodiments, the surface for immobilization of chaperoinin is a protein chip or a plurality of beads.

In yet another embodiment, the captured low molecular weight proteins have molecular weights less than 50 KDa.

Preferably, according to the second aspect of this invention, we provide a kit comprising a surface having a chaperonin disposed thereon to capture low molecular weight proteins of a denatured fluid sample.

We provide according to the third aspect of this invention the use of entrapping low molecular weights for diagnosis of hepatocelluar carcinoma, Alzheimers' disease, bladder and kidney diseases, and breast cancer in various embodiments.

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1:** Capture and release cycle of serum proteins by bacterial chaperonins.

**Figure 2:** Capture of serum proteins by Gro ELS under different conditions.

## DETAILED DESCRIPTION OF INVENTION

It is proposed that protein aggregates are firstly dissociated by denaturing followed by molecular cage, ie. bacterial chaperonin.

The advantages are manifold: 1) GroELS complex capture proteins that are smaller than 50 kDa. So serum albumin and other most abundant proteins will not be captured. This is a big advantage for normalizing protein concentration of different protein species in subsequent downstream analysis. 2) Cycles of capture and release lead to dissociation of protein from complexes such as those with serum albumin, eventually leading to complete capture of minor proteins in serum (Figure 1). Complete capture of minor proteins herein refers to possible means of concentrating minor protein/s of interest This approach enhances the possibility of finding potential significant biomarkers associated with or derived from early stages of diseases.

GroELS substrate specificity is promiscuous and current data suggest that substrate recognition involves the apical domain (Xu *et al.*, 1997) of the protein in association with several factors contributed by the substrate in question. These factors include 1) its exposed hydrophobic domain/s (Wang *et al.*, 1999); 2) its

extended ß-stands and a-helices with exposed side-chains (Jean Chatellier *et al.*, 1999), 3) its amphipathic secondary structures (Brazil *et al.*, 1997) and 4) its molecular size for proper encapsulation within the central cavity of GroEL (Sakikawa *et al.*, 1999). Taken together, the plasticity of GroELS machinery for substrate binding is in itself elegance and its substrate range *in vitro* is presently inchoate. To this end, the utilization and engineering of GroE architecture for the capturing of proteins from complex mixture such as human serum will generate invaluable information with respect to biology as well as biotechnology.

## Materials and Methods

Serum was derived from normal human sample while the Protein chip system is based on Ciphergen ProteinChip system and all other reagents were from Sigma. Although human serum is used in this initial study, other samples include but not limited to cerebral spinal fluid for Alzheimer's disease, urine for bladder or kidney diseases and nipple aspirant for breast cancer.

PS1 Chip preparation for GroEL immobilization: Standard protocol for PS1 chip application is available from Ciphergen. Briefly spots on PS1 chip were rehydrated in the recommended buffer, upon removal of the buffer the spots were added with 3µl of GroEL (2µg/µl), and the chip was incubated overnight in a humidifier at 4°C. These procedures will permit approximately 1pmole of protein of interest to be immobilized on the chips.

Human serum denaturation and capturing of serum proteins by GroELS: 25 µl of denaturation buffer was added to 25 µl of normal human serum (denaturation buffer composition: 100mM Tris-HCl pH 7.4, 2 mM EDTA, 20mM dithiothreitol (DTT), final concentration of guanidine hydrocholoride was maintained at 6 M). And denaturation was performed at room temperature for 1 hour. At the end of the denaturation period the serum was diluted 50 fold in renaturation buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl, ± 2mM ATP). This diluted serum was subsequently allowed to interact with GroEL immobilized on PS1 chip using a bioprocessor (Ciphergen). Interactions between GroELs and serum proteins under different conditions (see Figure 2) were conducted at room temperature for 4 hours. At the end of this period spots on chip were processed as prescribed in the standard protocol (Ciphergen) prior to analysis by ProteinChip Reader.

Clear-cut signals were not obtained in this preliminary proof-of-principle experiment, mainly due to the fact that the released proteins by the GroELS system are not being separated from the chaperonin within the bioprocessor. Therefore, if these proteins are not GroELS natural substrates, the tendency that they are not folded correctly is high. In other words, the released proteins can once again be captured by GroELS under appropriate conditions. Nevertheless

the results herein support the concept that GroELS can be utilized to capture serum proteins with distinct characteristics.

Site directed mutagenesis was employed to bioengineer GroEL for the desired functions, which are not natural to the wild type. The intended mutations designed for GroEL wild type are as follow and are based on the crystal structure of GroEL (Xu *et al.*, 1997):

1) Mutagenesis of GroEL protein sequence to enable proper orientation and least stereo-hindrance on solid surface for immobilization

Asp-490 ? Cys. Expected functional changes will be the introduction of an opened thiol (-SH) group at the equatorial domain of the protein. The –SH group introduced will facilitate the introduction of biotin at this site, thereof subsequent immobilization of GroEL on beads/matrix by means of biotin and streptavidin interaction can be achieved. Similar immobilization methods had been demonstrated previously (Taguchi *et al.*, 2001). The capturing capacity can be increased by immobilizing chaperones on beads, which can be packed into columns.

2) Mutagenesis of GroEL protein sequence by specific modifications made to the apical domain of GroEL to narrow or broaden the spectrum of the captured proteins.

Leu-200 ? Arg, Ser-201 ? Tyr, Pro-201 ? Asp. This mutation will generate a new apical domain with the introduction of RYD motif. The RYD sequence is a ubiquitous sequence recognized by cell surface receptors called integrins, which mediate and coordinate cellular responses to the extracellular matrix (ECM). Hence, they indirectly participate in cellular signaling pathways (Kinbara *et al.*, 2003). These modifications to GroEL will anticipate capturing of proteins within the human serum involving in signal transduction in a gross sense.

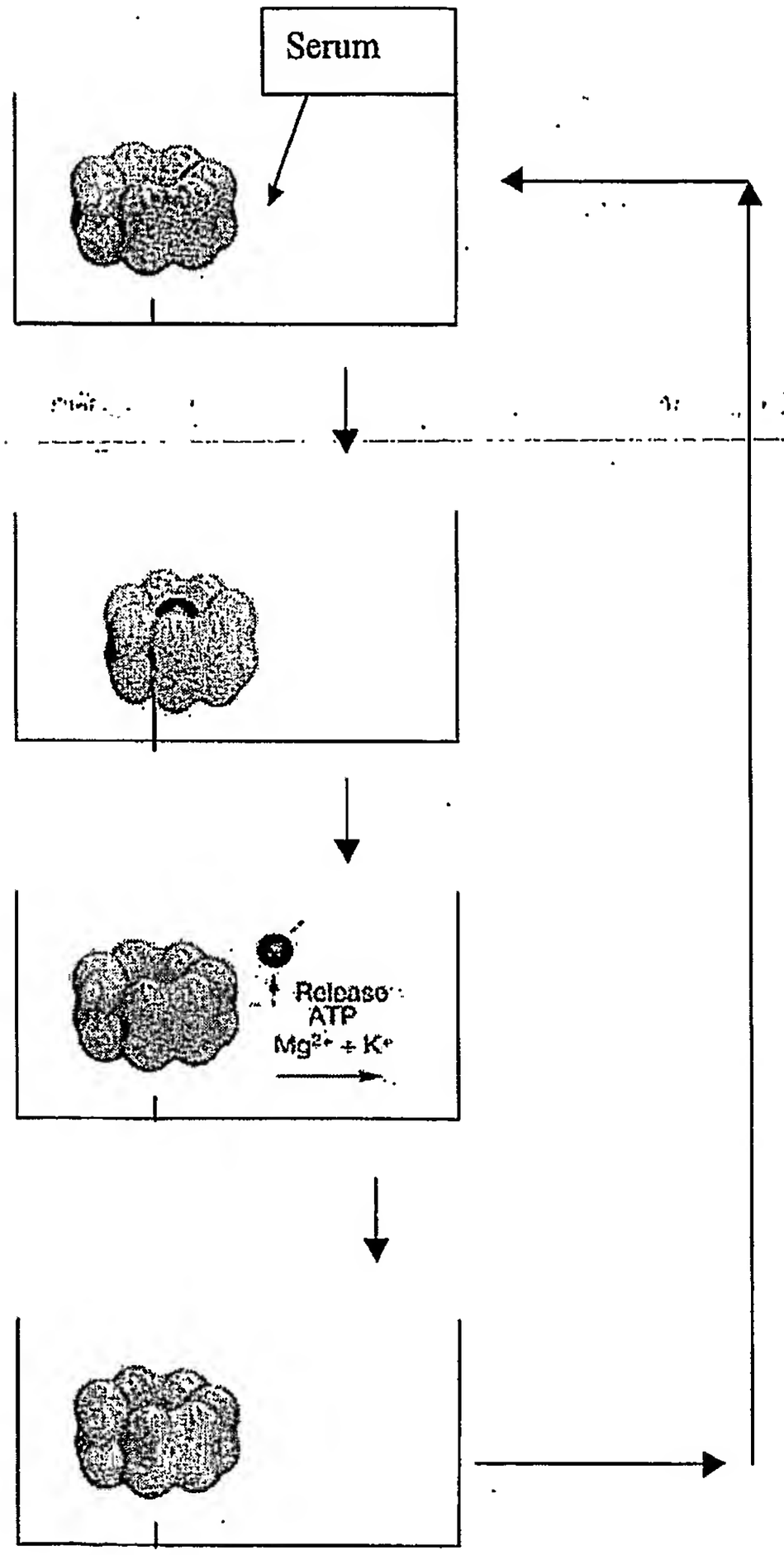
3) Mutagenesis of GroEL protein sequence to recognize different groups of proteins

Mutant Tyr-199 ? Ile, Tyr-204 ? Ile, Leu-234 ? Ile, Leu-237 ? Ile, Leu-259 ? Phe, Val-263 ? Leu, Val-264 ? Phe. These modifications involved the replacement of the substrate binding domain of GroEL (Group I chaperonin) with the substrate binding domain of Thermosome (Group II chaperonin) (Klumpp *et al.*, 1997). Such modifications may be lethal to the host or it may result anticipatively in the capturing of Group II chaperonin substrates. Consequently, the objective is to profile selective proteins with at least 200 species in a patient as a method of diagnosis.

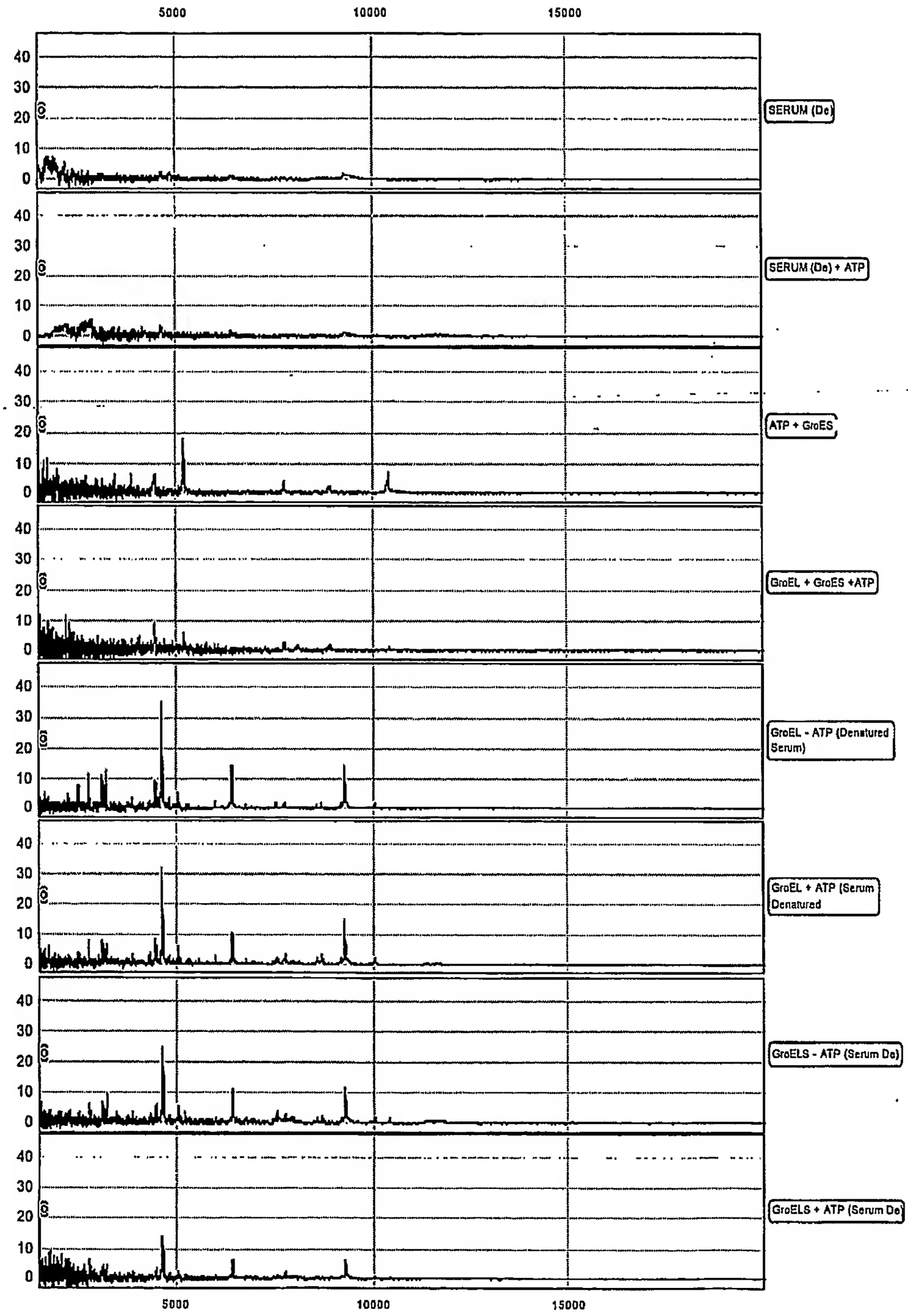
## EXAMPLES

On panels 1-4 of the figure 2, it suggests that serum proteins and serum protein in the presence of ATP did not bind significantly to the PS1 chip. GroES in the presence of ATP alone (panel 3) may generate a few non-specific peaks besides the GroES monomer as observed. In panel 5, GroEL captured a range of denatured serum proteins/peptides with molecular weight between ~ 2500 Da to 10,000 Da especially with one dominant species appearing at a molecular weight of 4654 Da (di-charge species at 9304 Da) under the prescribed experimental conditions. In panel 6, when ATP was added the intensity of the captured proteins/peptides appeared to be attenuated, this is in accordance with the current opinion that GroEL releases its captured substrate by the hydrolysis of ATP (Hartl and Hayer-Hartl, 2002). In panel 7, taking into consideration the dominant species the presence of GroES attenuated its intensity further. And finally in panel 8 the presence of both GroES and ATP reduced the overall intensity of captured proteins with respect to panel 5. These results are consistent with the known functional mechanistic of GroELS system (Sakikawa et al., Hartl and Hayer Hartl, 2002).

**Figure 1:**



## Figure 2:



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**WE CLAIM:**

1. A method of entrapping low molecular weight proteins comprising:

   immobilizing a chaperonin on a surface;
   and
   capturing low molecular weight proteins of a denatured fluid sample with the chaperonin.

2. The method of Claim 1, further comprising modifying the chaperonin by site-directed mutagenesis.

3. The method of Claim 2, wherein the modification comprises modifying apical domain of the chaperonin and thereby control spectrum of captured low molecular weight proteins.

4. The method of Claim 1, wherein the fluid sample is a serum.

5. The method of Claim 1, wherein the fluid sample is a cerebral spinal fluid.

6. The method of Claim 1, wherein the fluid sample is urine.

7. The method of Claim 1, wherein the sample is nipple aspirant.

8. The method of claim 1, wherein the chaperonin is *E. Coli* GroEL.

9. The method of claim 1, wherein the chaperonin is *T.th* GroEL.

10. The method of claim 1, wherein a protein chip provides the surface.

11. The method of claim 1, wherein a plurality of beads provides the surface.

12. The method of claim 1, wherein the captured low molecular weight proteins have molecular weights less than 50 KDa.

13. A kit comprising a surface having a chaperonin disposed thereon to capture low molecular weight proteins of a denatured fluid sample.

14. The kit of Claim 13, wherein the chaperonin is modifiable by site directed mutagenesis.

15. The kit of Claim 14, wherein the chaperonin is modifiable at apical domain to thereby control spectrum of captured low molecular weight proteins.

16. The kit of claim 13 wherein the sample is serum.

17. The kit of claim 13 wherein the fluid sample is cerebral spinal fluid.

18. The kit of claim 13 wherein the fluid sample is urine.

19. The kit of claim 13 wherein the fluid sample is nipple aspirant.

20. The kit of claim 13 wherein the chaperonin is *E. Coli* GroEL.

21. The kit of claim 13 wherein the chaperonin is *T.th* GroEL.

22. The kit of claim 13 wherein a protein chip provides the surface.

23. The kit of claim 13 wherein a plurality of beads provides the surface.

24. The kit of claim 13 wherein the captured low molecular weight proteins have molecular weights less than 50 KDa.

25. Use of the method of claims 1-3, 4 for diagnosis of hepatocellular carcinoma.

26. Use of the method of claims 1-3, 5 for diagnosis of Alzheimers' disease.

27. Use of the method of claims 1-3, 6 for diagnosis of bladder and kidney diseases.

28. Use of the method of claims 1-3, 7 for diagnosis of breast cancer.

## ABSTRACT

The invention describes a method of entrapping low molecular weight (<50Kda) proteins from fluid sample such as human serum using bacterial chaperonins immobilized on a surface. In particular, the bacterial chaperonins are Gro ELS proteins from *E.coli* or *T.th* (*Thermos thermophilus* HB8) while the solid surface comprises of either a protein chip or beads. Furthermore, it describes a method of modification of apical domain of the GroEL proteins to capture specific proteins and thereby identifying significant biomarkers in early stages of diseases such as Alzheimer's disease, bladder and kidney diseases and breast cancer.